86-C 5534

B4 C3 D10

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4: C12N 15/00, A61K 39/21, C12N 7/00 — A1

(11) International Publication Number: WO 86/00930

(43) International Publication Date: 13 February 1986 (13.02.86)

(21) International Application Number: PCT/US85/01352

(22) International Filing Date: 18 July 1985 (18.07.85)

(31) Priority Application Number: 632,723

(32) Priority Date: 20 July 1984 (20.07.84)

(33) Priority Country: US

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(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RETROVIRAL VACCINES AND VECTORS AND METHODS FOR THEIR CONSTRUCTION

(57) Abstract

Retroviruses and retrovirus vectors that either are or are not shed into eggs and therefore either do or do not undergo congenital transmission, and methods for making them. This invention is based on the discovery that the ability of exogenous avian leukosis viruses to undergo congenital transmission is determined by *gag* sequences of the viral genome.

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RNA AND DNA CONTG. SEQUENCES OF RETROVIRUSES ESP. AVIAN RETROVIRUSES + USEFUL AS VECTORS THAT ARE OR ARE NOT SHED INTO ECGS TO CONTROL CONGENITAL TRANSMISSION OF THE VIRUSES

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RETROVIRAL VACCINES AND VECTORS AND METHODS FOR THEIR CONSTRUCTION

Technical Field

This invention is in the fields of molecular biology and virology and in particular relates to the use of retroviruses for construction of vaccines and expression vectors.

Background Art

The Retroviridae virus family includes viruses which contain an RNA genome and an RNA-dependent DNA polymerase activity (reverse transcriptase). During their growth cycle, retroviridae, or retroviruses as they are more commonly called, copy their RNA into proviral DNA. Proviral DNA becomes inserted (integrated) into the chromosomal DNA of the host where it uses the transcriptional and translational machinery of the host to express viral RNA and proteins. Viruses are released from the cell by budding from the cytoplasmic membrane. Most infections do not kill cells. Rather, infected cells continue to grow and differentiate while continuously producing virus.

The Retroviridae family is divided into three subfamilies: 1) Oncovirinae, which include the oncogenic members and many closely related non-oncogenic viruses; 2) Lentivirinae or the slow viruses; and 3) Spumavirinae or "foamy" viruses, which induce persistent infection but no clinical disease. R. Weiss et al (ed.), Molecular Biology of Tumor Viruses (2d ed.) RNA Tumor Viruses, 26, Cold Spring Harbor Laboratory (1982).

Viruses in the three subfamilies produce widely varying effects in infected cells but have similar morphological, biochemical and physical properties. Common morphological characteristics include a lipid envelope surrounding an icosahedral protein shell or capsid, which in turn surrounds a ribonucleoprotein (RNP) complex or core (nucleoid). The capsid and the RNP core as a unit are called a nucleocapsid. Retroviruses have genomic RNA which is a dimer composed of two identical subunits and also have similar protein, lipid, carbohydrate and RNA compositions.

The RNA genome of all replication-competent retroviruses (those capable of producing fully infectious progeny) contains three genes which encode viral structural proteins. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase); and the env gene encodes the envelope glycoproteins of the virion. The genetic organization of all retroviruses is similar to that of avian leukosis virus (ALVs). The proteins encoded by the gag, pol and env genes of

members of the different retroviral families differ in size but serve similar functions.

If a genome lacks any one of these three genes, the virus in which it occurs is defective for replication. This defect may be circumvented if cells infected with a defective virus are coinfected or superinfected with a related virus that can serve as a helper virus. A helper virus provides proteins required by the defective virus to undergo an infectious life cycle. The proteins provided by the helper virus confer on the particles containing the genome of the defective virus the antigenic characteristics as well as the host range and growth restrictions of the helper virus.

In addition to the gag, pol, and env genes, all retroviral genomes, whether replication competent or defective, contain sequences encoding signals essential to the reverse transcription of viral RNA into proviral DNA; the integration of proviral DNA into host chromosomal DNA; the transcription of viral RNA and mRNA from integrated proviral DNA; the translation of viral mRNA into protein; and the packaging of viral RNA into virions. These sequences act only on the DNA or RNA in which they are contained and are therefore referred to as cis-acting signals. Cis-acting signals that are essential for an RNA to be able to undergo a retrovirus life cycle are found in a short repeat sequence (R) located at both the 5' and the 3' ends of viral RNA; in sequences that are unique to the 5' (U5) and the 3' (U3) ends of viral RNA; and in sequences lying immediately adjacent to U5 and U3.

In infected cells, viral RNA is reverse transcribed by the RNA-dependent DNA polymerase of the virus into proviral DNA. Proviral DNA is found in both integrated and unintegrated forms in infected cells. The unintegrated forms may be linear molecules or closed circular molecules. Integrated as well as unintegrated proviral DNAs have similar genetic organizations. In proviral DNA the R, U5, and U3 sequences of viral RNA are long terminal repeat (LTR) sequences that flank the gag, pol, and env genes. The LTRs, as well as immediately adjacent sequences, contain cis-acting signals that are essential for an RNA to be able to undergo a retrovirus life cycle.

The gag, pol and env sequences of the retroviral genome each encodes highly specific protein products. The gag gene product of ALVs is a polyprotein (approximately 65,000-80,000 daltons) In the avian retroviruses, the gag polyprotein is cleaved to produce four major core or capsid proteins: p19, p12, p27 and p15. p19 occurs in association with viral RNA and the lipid envelope and may have a role in viral mRNA metabolism. p12 occurs in association with viral RNA and is apparently the major protein of the viral RNP complex. p27 is thought to form the core shell or capsid which surrounds the RNP and lies between the RNP and the lipid envelope. p15 is a protease that cleaves the p19, p12, and p27 proteins from the gag-polyprotein. In addition, p10, a minor nonglycosylated polypeptide, is encoded by gag and is thought to reside on or within the viral envelope.

The pol gene product is the viral RNAdependent DNA polymerase responsible for the synthesis of proviral DNA on the viral RNA template. The gag and pol genes, as well as their protein products, are highly conserved among related strains of viruses.

The env gene encodes the envelope or surface glycoproteins of the virion. Related groups of retroviruses are classified into subgroups according to host range, serum neutralization, and interference patterns. Each of these properties is determined by the env-gene products. In ALVs, an env-gene polyprotein is cleaved to produce two envelope glycoproteins, gp85 and gp37. gp85 sequences contain the antigens that determine host range and elicit the production of neutralizing serum by the infected host. gp37 serves to anchor gp85 in the viral membrane. ALVs have been classified into 5 different subgroups, labelled A through E, on the basis of determinants found in gp85.

Retroviruses are distributed widely among vertebrates and may also occur in some invertebrates. Orders from which retroviruses have been isolated include Galliformes (chickens, pheasants, turkeys); Rodentia (rats, mice, hamsters, guinea pigs), Carnivora (cats); Artiodactyla (cattle, deer, pig); and Primates (monkeys, apes, humans). Many of these orders contain more than one family of retroviruses. For example, avian retroviruses fall into four groups, which include: the avian sarcoma and leukosis viruses of chickens (ALVs); the pheasant viruses of pheasant and quail; the reticuloendotheliosis virus of turkeys; and the lymphoproliferative disease viruses of turkeys.

Most families of retrovirususes exist and are transmitted by endogenous as well as exogenous viruses. Endogenous retroviruses are retroviruses whose proviral DNA has become inserted into the germ line DNA of their host. These viruses are endogenous to their host in the sense that they have become part of the genetic information of their host. Although endogenous viruses can be transmitted by infections, the most frequent route for the transmission of these viruses is through the proviral DNA present in gametes.

Exogenous retroviruses are viruses whose genetic information is being transmitted exclusively by virus infections. The vast majority of these infections do not result in integration of proviral DNA into the DNA of gametes. Thus these infections are not transmitted by the gametes of their hosts.

The structure of endogenous proviruses is the same as that of proviruses that result from infection by an endogenous or exogenous virus. However, endogenous members of a group of retroviruses frequently have genes or control signals that encode slightly different biological properties from those found in exogenous ALVs. These differences in homologous sequences determine differences in the disease potential and transmission of endogenous and exogenous virus infections.

Exogenous viruses are associated with a wide spectrum of diseases which vary in incidence and time of onset. For example, exogenous ALVs cause B-cell lymphomas, erythroid and myeloid leukemias, hemangiomas, and osteopetrosis. In most instances, only a fraction of the infected birds develop

disease. For example, in the case of erythroblastosis, disease occurs only if a provirus inserts into or becomes a transducing agent for the host gene c-erbB. However, even if overt disease does not develop; infected birds, especially those that become persistently viremic, tend to lag behind uninfected controls in weight gain and egg production.

In contrast, infections by most endogenous viruses tend to be benign. When endogenous ALVs are introduced back into chickens as infections, no disease is observed and infected chickens appear to undergo normal weight gain and egg production.

In order to survive, exogenous viruses must undergo efficient transmission from infected to uninfected hosts. In most instances, exogenous viruses have evolved so that they undergo highly efficient transmission by congenital and neo-natal infections. Congenital and neonatal infections, because they occur in hosts whose immune systems are not fully developed, tend to establish viremias that persist throughout the lives of the infected animal. For most exogenous viruses, congenital and neonatal infections provide the major reservoirs of virus in infected populations. Thus, control of congenital infections is key to the erradication of a retrovirus.

For example, ALVs of exogenous origin replicate throughout the reproductive tract of the hen, with large numbers of budding virus occurring in the magnum- or albumen secreting region of the oviduct. The developing embryos become infected and hatch into chickens whose immune system recognizes the

virus as self. Because the ALV antigens are recognized as self, antibodies against ALVs are not made. A persistent viremia is established and each generation of viremic hens produces viremic offspring.

Domesticated animals that are raised in flocks or herds (e.g., poultry, cattle, sheep) are particularly vulnerable to retrovirus infections. Centralized breeding programs and crowded conditions of commercial farms favor the persistence and spread of retrovirus infections. For example, avian leukosis virus infections in laying hens are a commercial problem because they increase mortality and decrease egg production. In addition, viremic hens shed virus into their eggs and therefore produce viremic progeny whose health and egg production are compromised by the infection.

The present approach by cattle, sheep and poultry farmers to the control of retrovirus infections is to eliminate viremic individuals from the breeding population. Although this approach has proved effective when progeny of virus-free individuals have been reared in isolation (e.g., in research facilities), it is far less effective when used on the farm. On the farm, identification of shedders may not be adequate and progeny of virus-free individuals become infected because they are raised in proximity to infected adults. Thus the infections persists and continue to be transmitted from one animal to another.

In the poultry industry, efforts have been made to control ALV infections by using field strains of ALVs as live vaccines. For example, Dutch

researchers have controlled ALV infections by eliminating shedders from their breeding population, rearing virus-free chicks in isolation until two months of age and then vaccinating the two-month old chicks with a mixture of subgroup A and subgroup B ALVs (RAV-1 and RAV-2). After this protocol was repeated for three generations, the flocks tested appeared to be free of ALV-associated disease. B. H. Rispens et al., "A method for the control of lymphoid leukosis in chickens," Journal of the National Cancer Institute 57: 1151-1156. (1976). The success of the Dutch researchers is due to the elimination of infected individuals from the breeding population, the availability of facilities to rear chicks in isolation, and the vaccination of birds at an age when the immune response to the infection curtails both the pathogenic potential of the virus as well as the infection of the oviduct and subsequent congenital transmission of the unattenuated vaccine virus.

The approach of the Dutch, however, is not suitable to commercial application because of the need for rigorous screening for hens that are shedding virus and for the isolation of chicks. Furthermore, the currently used method of controlling ALV infections in the U.S. --elimination of shedders-- is inefficient, because it fails to identify many viremic hens, and expensive because of the tests required to identify shedders and the requirement that shedders be eliminated.

Summary of the Invention

This invention arises from the Applicant's

discovery that the ability of exogenous avian leukosis viruses to undergo congenital transmission is determined by the gag sequences of the viral genome. A direct correlation exists between the presence of sequences encoding the gag-encoded p27 protein of exogenous ALVs and the ability of a virus to be shed into egg albumen. That is, recombinants of endogenous and exogenous ALVs with the exogenous p27 marker of the exogenous parent undergo efficient replication in the oviduct and shedding into eggs, but those with the analogous marker (p27°) from the endogenous parent do not.

This knowledge has made it possible to construct retroviruses and retrovirus vectors that either are or are not shed into eggs and therefore either do or do not undergo congenital transmission. Retroviruses and retrovirus vectors constructed for use as vaccine viruses would be attenuated viruses that contain the gag sequences of an endogenous retrovirus and thus are not shed into the egg. Therefore, congenital infections do not occur. On the other hand, retroviruses constructed according to the method of this invention which are to be used for obtaining desired proteins in eggs contain the gag sequences of an exogenous retrovirus. As a result, the virus infects the oviduct and the products of the vector virus are shed into the egg.

In one embodiment of this invention, benign (nonpathogenic) but immunizing vaccines against ALVs are made. The vaccine viruses are constructed from nucleic acid sequences which include sequences from endogenous and exogenous ALVs. The vaccine viruses

are constructed so as to express gp85-host range determinants of the exogenous virus or viruses against which the vaccine is directed. These host range or type specific determinants encode antigens which elicit an immune response to the virus. The vaccine viruses have the gag sequences of an endogenous ALV. The gag sequences of endogenous ALVs ensure that the vaccine virus will not undergo congenital infections or transmission. The vaccine viruses have pol and LTR sequences from ALVs of endogenous or exogenous origin and may have sequences not found in endogenous or exogenous ALVs.

The DNAs which contain sequences from endogenous and exogenous ALVs are used as vaccines by either directly inoculating DNA into chickens Fung et al Proceedings of the National Academy of Sciences, 80:353-357 (1983) or by inoculating virus expressing the DNA following transfection of culture cells Graham et al, Virology, 52:456-467, (1973) as modified by Loyter et al, Proceedings of the National Academy of Sciences, 79:422-426(1982). The vaccine of this invention is unique in that it evokes the immune response necessary to immunize the chicken against an exogenous ALV infection but produces no pathogenic effects in the recipient and is not shed into the egg. As a result, adults will be immunized against an exogenous ALV infections and progeny of vaccinated hens will not be tolerized to the exogenous ALV.

In another embodiment of the method of this invention, vaccines for viruses other than retroviruses are made. In this embodiment, the vaccine virus consists of a vector virus and a

helper virus. The vector virus is a replication defective retrovirus that expresses antigens encoded in the nucleic acid sequences of another virus. That is, it contains sequences encoding determinants that stimulate the production of neutralizing serum to the virus against which an immune response is desired. The vector virus includes sequences encoding the desired antigens as well as sequences from endogenous and exogenous ALVs. The helper virus is a retrovirus which provides the necessary proteins for the replication of the vector virus. The helper virus is a benign virus made from nucleic acid sequences which include those of endogenous and exogenous ALVs. In particular it contains the gag sequences of endogenous virus that restrict the replication of the vector as well as helper virus in the oviduct.

Virus expressed by cultured cells that have been contransfected with vector and helper DNAs are used as inocula for vaccination. A mixture of the vector and helper DNAs may also be used as an inocula. The vaccine made according to this method immunizes against a virus other than a retrovirus but because the helper virus does not replicate in the oviduct, progeny of vaccinated hosts will not be tolerized to antigens expressed by the vector or helper components of the vaccine.

In still another embodiment of the method of this invention, retrovirus stocks that serve as expression vectors for specific proteins are made. These expression vectors make it possible to have a desired protein secreted at high levels into the eggs of infected hens. The virus stock consists of

a vector virus and a helper virus. The vector virus is a replication defective virus which expresses the protein to be secreted into egg albumen under the transcriptional control of sequences that normally control the transcription of chicken egg white protein. The vector is made from sequences that include: 1) sequences encoding the desired protein, 2) sequences containing control signs for egg white protein production, and 3) sequences from endogenous and exogenous ALVs. The helper virus is a retrovirus which provides the proteins necessary for the vector virus to establish efficient infection of the oviduct. It is made from sequences that include those from endogenous and exogenous retroviruses. In particular, the helper contains the gag sequences from an exogenous retrovirus that ensure efficient infection of the oviduct by the vector virus. Virus expressed by cultured cells that have been contransfected with vector and helper DNAs are used as inocula. The virus stocks in this embodiment are unique in that they result in proteins of commercial value, such as hormones, blood clotting factors and plasma proteins being secreted into the eggs. The secreted protein is subsequently harvested from the egg.

Brief Description of the Figures

Figure 1a is a simplified representation of the viral RNA of an ALV; Figure 1b is a simplified representation of a linear form of proviral DNA of an ALV.

Figure 2 is a simplified representation of DNA sequences used in making a vaccine for ALVs

according to this invention, wherein: Figure 2a is a simplified representation of the DNA of the endogenous ALV, RAV-0; Figure 2b is a simplified representation of the DNA of the exogenous ALV, RAV-1; and Figure 2c is a simplified representation of the DNA of a construct used as a vaccine or for the recovery of a vaccine virus.

Figure 3 is a simplified representation of DNAs used in making retroviral vaccines for viruses other than retroviruses according to this invention, wherein: Figure 3a is a simplified representation of a DNA sequence that encodes an antigen (or antigens) of a virus other then a retrovirus. Figure 3b is a simplified representation of the DNA of a helper virus that has been made from sequences present in endogenous and exogenous ALVs; and Figure 3c is a simplified representation of the proviral DNA of a retroviral vector that expresses the antigens of 3a.

Figure 4 is a simplified representation of the DNAs used in making a retrovirus stock that results in a desired protein being secreted into eggs of infected hens according to this invention, wherein: Figure 4a is a simplified representation of a DNA sequence that controls the expression of ovalbumin; Figure 4b is a simplified representation of a DNA sequence that encodes the protein to be secreted into egg albumen; Figure 4c is a simplified representation of the proviral DNA of a helper virus, made from sequences found in endogenous and exogenous ALVs;and Figure 4d is a simplified representation of a retroviral vector that expresses the protein encoded in the DNA sequence shown in

Figure 4b under the transcriptional control of sequences shown in 4a.

Best Mode of Carrying Out the Invention

The ability to make a retrovirus which is or is not transmitted by congenital infection is based on the discovery that the shedding of ALVs into the eggs of laying hens is determined by the gag sequences of the viral genome. A direct correlation has been shown to exist between the presence of the gag-encoded p27 protein of exogenous ALVs and the ability of a virus to be shed into egg albumen. Viruses with the exogenous p27 protein were shown to undergo efficient shedding into eggs, whereas viruses with the endogenous p27° protein were shown not to be shed into eggs.

This discovery is the result of the examination of the shedding into eggs of eight recombinants of endogenous and exogenous ALVs. This examination was carried out in an effort to define the region of the genome of endogenous viruses which restricted the shedding of endogenous ALVs into the eggs of K28 hens.

K28 is a line of chickens bred for susceptibility to endogenous virus infections. Endogenous viruses replicate well in K28 chickens, establishing levels of viremia comparable to levels observed in K28 chickens infected with exogenous ALVs. K28 hens infected with exogenous ALVs shed high titers of virus into egg albumen but those infected with the endogenous virus RAV-0 did not shed virus into egg albumen.

The recombinants examined included: 1) four viruses (RAV-60s) which were generated during growth of exogenous viruses in cells that expressed replication-defective endogenous viruses; two viruses (NTRE-2 and NTRE-7) which were generated during mixed infection of cells with an exogenous virus and an endogenous virus; and three viruses (recALVWF44,45 and recALVBr22) which were recovered from DNA constructions that recombined specific fragments of molecularly cloned proviral DNAs.

Tests for the transmission into eggs of the recombinant viruses were performed on eggs layed by viremic K28 hens. The presence of virus in egg albumen was assayed by incubating albumen with antiserum to the group specific antigens of ALVs and then testing for complement fixation.

Five of the eight recombinant viruses underwent efficient egg transmission; whereas, three did not. The genomes of the recombinants contained a number of markers for sequences from their endogenous and exogenous parents. Most of the markers were oligonucleotides that were diagnostic for the RNA of the endogenous or the exogenous parents. The host range of the recombinant was used as a marker for gp85 sequences. (gp85 sequences contain the type specific antigens which determine the host range of a retrovirus.) The electrophoretic mobility of the p27 protein was used as a marker for the sequence which encodes it.

All eight of the recombinants had the subgroup E host range of their endogenous parent and U_3 sequences from their exogenous parent. Thus, sequences encoding host range and U_3 do not

determine whether ALVs undergo efficient congenital transmission. In addition, markers in the U5, p19, and gp37 sequence from the endogenous and exogenous parents did not correlate with the ability of the recombinant to undergo congenital transmission. Thus, these sequences do not play a role in congenital transmission.

However, each of the five recombinants which underwent efficient shedding into eggs had the p27 marker of the exogenous parent. Conversely, the three recombinants which did not undergo efficient shedding had the p27° marker of the endogenous parent. Thus, there is a direct correlation between the presence of the p27 marker of exogenous ALVs and the ability to be shed into egg albumen.

The complement fixation assay for gs antigens is a relatively insensitive assay for ALVs. Therefore, the restriction on the congenital transmission of endogenous ALVs was examined by culturing embryonic progeny of hens infected with RAV-0 or NTRE-7. RAV-0 is the prototype endogenous virus. NTRE-7 is a non-transmitted recombinant that has the high growth potential of exogenous ALVs. Cultured cells were assayed for virus at one, two and three weeks of culture. Since, in this period of time, even a single infectious unit of virus would have had ample time to spread throughout the culture and thus score in the assays for virus, the restriction on the ability of RAV-0 and NTRE-7 to be passed by congenital infection in K28 chickens is absolute. Nucleic acid sequences encoding viruses may be made from (constructed) sequences found in endogenous or exogenous viruses. The sequences can

be obtained from viral RNA or DNA. Synthesized DNA sequences not occurring in viruses may also be used.

I. Vaccine Viruses for Retroviruses

Based upon the aforementioned findings, attenuated viruses can be made for the control of exogenous ALV infections. The vaccine viruses, which are benign but immunizing, can be made from the DNAs of endogenous and exogenous ALVs. The vaccines contain DNAs or ALVs expressed by the DNA following transfection of cultured cells with the DNAs. The DNAs contain sequences that encode antigens of an exogenous ALV and the gag sequences of an endogenous ALV, such as RAV-0. The gag sequences of RAV-0 restrict the replication of the vaccine virus in oviduct tissue and ensure that the vaccine virus is not transmitted to the progeny of vaccinated hens by congenital infections. If congenital infections were not controlled, the vaccine virus would be transmitted to the developing chick, which would recognize it as "normal" and fail to respond the antigens expressed by the vaccine. Use of the virus as a vaccine is superior to the use of a field strain of an ALV as a vaccine because the constructed virus is benign, and does not tolerize progeny of the vaccinated adult to the vaccine.

Figure 2 illustrates the construction of such a vaccine. Figures 2a and 2b are simplified representations of DNA sequences used in making the vaccine. pRAV-0 (Figure 2a) and pRAV-1 (Figure 2b) represent, respectively, the DNAs of RAV-0 (an

endogenous retrovirus) and RAV-1 (an exogenous ALV of subgroup A). pA (Figure 2c) represents DNA of a construct used as a vaccine or for the recovery of a virus for use as a vaccine. pA is made by linking DNA sequences comprising the DNA of pRAV-0 and pRAV-1. The sequences used in pA may be synthesized or may be generated from proviral DNAs by the use of nucleases. Where necessary, DNA fragments are amplified by growth in plasmid vectors.

pA contains gag and pol sequences of pRAV-0 and env sequences of pRAV-1. The pRAV-1 env sequences which are substituted for the pRAV-0 env sequences encode the type specific determinants of the gp85 of pRAV-1. The type specific antigens of gp85 determine host range and elicit an immunizing response to infections by subgroup A viruses. gag sequences of RAV-0 ensure restricted replication in oviduct tissue. Because viral replication in the oviduct is restricted, the vaccine virus will not be shed into eggs and the progeny of vaccinated hens will not be tolerized to the vaccine virus. pol sequences from pRAV-0 are used because RAV-0 is a benign virus. LTR sequences of RAV-0 that encode an intermediate growth potential have been used in pA.

Construction of a vaccine for subgroup A ALVs is described in detail in Example 3. The vaccine constructed as described in the example was shown to have most of the gag and all of the pol sequences of the benign RAV-0 virus and the env and the LTR sequences of the subgroup A RAV-1 virus.

Sequences that encode higher growth potentials could be used instead of those from RAV-0. ALV vaccines with sequences encoding other antigenic determinants can be made using sequences encoding

the host range of other ALVs such as Resistance Inducing Factors (RIF), helpers for laboratory strains of acute sarcoma and leukemia viruses (e.g. Rous Associated Viruses (RAVs)) and transformation defective derivatives of acute transforming viruses (e.g., td Praque Rous Sarcoma Virus (tdPrRSV)).

Virus for the vaccination of chickens is expressed by DNA following cleavage of the DNA from vector plasmids and transfection of cultures of eukaryotic cells, such as turkey or chicken. Viruses recovered from the transfected cultures are propagated by infection of eukaryotic cells, such as turkey, chicken or quail. Chickens can also be vaccinated by inoculation of the DNA.

The method of this invention can also be used to made vaccine viruses from retroviruses other than avian retroviruses. For example, endogenous and exogenous feline leukemia viruses could be used for the construction of a vaccine for feline leukemia virus. The endogenous viruses used can be proviruses encoding replication competent as well as replication defective feline leukemia viruses. The exogenous viruses, against which immunization is desired, are field strains of feline leukemia viruses and helper viruses for the acute leukemia and sarcoma-inducing viruses of cats. gag sequence of the endogenous virus may restrict replication in the reproductive as well as mammary and salivary tissues; which are important sources of virus for the congenital and neonatal infection of new born cats. In this embodiment one would not necessarily be limited to using sequences from the same family of retroviruses or sequences originally observed in retroviruses for the construction of vaccine

viruses. For example, a suitable vaccine for bovine leukemia viruses might be constructed with sequences found in bovine leukemia viruses as well as gag sequences found in feline or murine leukemia viruses. Or, gag sequences observed in bovine leukemia viruses could be appropriately modified in vitro so that these sequences, when used in constructs would provide benign yet immunizing infections that are not readily transmitted to embryos or neonates.

II. Vaccine Viruses for Viruses Other Than Retroviruses

In another preferred embodiment of this invention, retroviruses are used to make retroviral vaccines for viruses other than retroviruses. The vaccines consist of two viral components: a vector virus and a helper virus. The vector virus is a replication defective retrovirus which expresses antigenic determinants of a virus other than a retrovirus. The helper virus is a retrovirus that provides the necessary proteins for the replication of the vector virus. Both vector and help viruses are made from nucleic acid sequences (e.g. DNA).

Avian diseases for which immunizing retroviruses could be made include: Marek's disease, for which the viral protein with immunizing determinants are envelope antigens; and avian influenza or fowl plague and Newcastle Disease, for which the viral protein with immunizing determinants is hemagglutinin. Marek's disease is caused by a herpes virus, avian influenza or fowl plague by orthomyxoviruses, and Newcastle Disease by a paramyxovirus.

Figure 3 illustrates an embodiment of this invention.

pX (Figure 3a) is a sequence that encodes an antigen or antigens which elicit an immune response to the virus from which pX was taken. The size and genetic content of pX depend upon the antigens which it encodes. pA (Figure 3b) is an example of a helper virus used to make as well as the propagation of vaccines for viruses other than retroviruses. pA is a representation of the DNA of an ALV made from RAV-0 and RAV-1 sequences (see Figure 2). It has gag and pol sequences from RAV-0 and env sequences from RAV-1. The gag sequences from RAV-0 restrict the replication of the virus encoded in the oviduct and as a result, the virus encoded in pA as well as any vectors for which it is a helper will not be shed into eggs. Therefore, adults will be immunized by the vaccine but progeny of vaccinated hens will not be tolerized to antigens expressed by the vaccine pol sequences from RAV-0 are retained because RAV-0 is a benign virus. The env sequences from RAV-1 encode a subgroup A host range. This host range ensures infection and therefore vaccination of the vast majority of egg laying stocks of chickens. gp85 sequences encoding other host ranges, for example that of RAV-2 which encodes subgroup B host range, could be used. pA has the LTR sequences of RAV-0 that encode an intermediate level of replication. LTR sequences that encode higher growth potentials could also be used to construct vaccine virus.

pAX (Figure 3c) is a representation of a DNA of a retroviral vector that expresses sequences in pX. As shown, pAX expresses antigens from only one DNA sequence, those in pX. pAX could be constructed

with DNA sequences expressing more than one antigen. The retroviral sequences in pAX have cis-acting signals for the reverse transcription of viral RNA into proviral DNA; the integration of proviral DNA into chromosomal DNA; the transcription of RNA from proviral DNA; and the packaging of transcripts of viral RNA into infectious virions.

Other sequences in pAX are those that facilitate the constructions while ensuring expression of the antigens encoded in pX and the transmission of the RNA expressed by the vector as a retrovirus.

pAX is made by linking pX sequences with DNA sequences of a helper virus such as that represented in pA. In pAX, pX sequences may be added in the same or the opposite transcriptional orientation to proviral sequences. Junctions of pA and pX sequences ensure expression of the antigens expressed in pX. Junctions also ensure that RNAs expressed by pAX undergo a retrovirus life cycle. DNAs other than those in pA and pAX may be used to make pAX.

Virus is recovered from DNA by cleavage of construct DNAs from plasmid vectors and the transfection of cultures of appropriate eukaryotic cells (e.g., turkey, chicken) with DNA. Vector DNAs are co-transfected with helper DNAs. Once a virus stock is obtained it is further replicated by infection of cultured cells. Animals can also be vaccinated by direct innoculation of a mixture of the vector and helper DNAs.

III. Retroviruses to be Used as Expression Vectors

In another preferred embodiment of this invention, retroviruses stocks are made that secrete

high levels of a desired protein into eggs. These stocks consist of an expression vector and a helper virus. The expression vector is made to maximize the secretion into eggs of a protein or proteins encoded in the vector. The helper virus is made so as to ensure an efficient yet non-pathogenic infection of the oviduct by the vector.

Figure 4 is a representation of the DNAs to be used in making a retrovirus stock which results in the secretion of high levels of a protein into the eggs of infected hens. The vector virus is a replication defective retrovirus which contains one or more genes whose products are to be secreted into the egg. The protein to be secreted into egg albumen is under the transcriptional control of sequences that normally control the transcription of a chicken egg white protein.

Products which could be expressed by the vector viruses include hypothalmic and pituitary hormones (e.g., thyroid releasing hormone, somatostatin); blood clotting factors (e.g., Factor VIII); plasma proteins (e.g., human serum albumin); peptide growth hormones (e.g., interleukins 1,2,3, insulin); anti-viral proteins (e.g., interferon); and secreted proteins (e.g., lipase).

The helper virus is a retrovirus which provides the necessary proteins for the vector virus to establish an efficient infection of the oviduct. The helper virus has the *gag* gene of an exogenous retrovirus, such as RAV-1, which ensures infection of the oviduct. *Pol*, *env* and LTR sequences of the helper virus are derived from endogenous retrovirus sequences such as those of RAV-0, or from exogenous

retroviruses. They ensure that the infection of the host chicken and oviduct is efficient but non-pathogenic.

pOV (Figure 4a) represents a DNA sequence that contains signals for the expression of an egg white protein (e.g., ovalbumin). The pol sequences ensure high levels of synthesis of pY in the oviduct and and that the RNA of the pCOVY vector is able to undergo a retrovirus life cycle.

pY (Figure 4b) represents sequences that encode the protein to be secreted into egg albumen. The sequence content of pY will depend upon the protein it encodes.

pC (Figure 4c) represents a helper virus used to make as well as propagate vectors containing pY sequences under the transcriptional control of pOV. pC is DNA of an ALV made from RAV-0 and RAV-1 sequences. pC has gag sequences from RAV-1 that ens re efficient infection of oviduct tissue. It also has pol sequences from the benign endogenous virus RAV-0. Env sequences are those from RAV-1 that encode a subgroup A host range. If other host ranges are desired, appropriate env sequences from other subgroups of ALVs can be used. pC has the LTR sequences of RAV-0 that encode an intermediate growth potential. Sequences that encode higher growth potentials could also be used in the construction of pC.

pC is made by linking sequences from RAV-0 and RAV-1 DNAs. Other DNAs could be used.

pCOVY (Figure 4d) represents a retroviral vector made from pC, pOV, and pY. This vector expresses the protein encoded in pY under the

transcriptional control of sequences in pOV. The sequences from pOV are those that give high levels of expression of proteins encoded in pY yet allow the RNA encoded by pCOVY to undergo a retrovirus life cycle. The expression of Y by pOV is such that it does not interfere with egg production by infected hens.

pCOVY is made by the linking of sequences from pOV, pY and pC. pOV and pY may be inserted into proviral DNA in the same or the opposite transcriptional orientation as proviral DNA. The retroviral sequences in pCOVY are those that contain cis-acting signals for the reverse transcription of viral RNA into proviral DNA; the integration of proviral DNA into host chromosomal DNA; the transcription of proviral DNA into RNA; and the packaging of proviral RNA into infectious virions. Other retroviral sequences in pCOVY include those that facilitate the preparation of the constructions while ensuring high levels of pY expression under the control of pOV sequences and the transmission of vector RNAs in infectious virions. Other DNAs may be used to make pCOVY.

Virus stocks are produced by cleaving helper and vector DNAs from plasmid vectors and co-transfecting appropriate cultures of eukaryotic cells, such as turkey, chicken. Once a virus stock is obtained it is propagated by infection of appropriate cultures of eukaryotic cells.

The method of this invention is not limited in application to retroviruses of avian origin. Retroviruses are found widely in nature and it is anticipated that retroviruses of feline, bovine and

murine origin, among others, can be used to construct retroviruses useful as vaccines and expression vectors.

Examples

Example 1: Congenital Transmission of Exogenous Avian Leukosis Viruses But Not Endogenous Avian Leukosis Viruses in K28 Chickens.

K28 chickens were intravenously inoculated at 1 day of age with approximately 1 x 10^6 infectious units (i.u.) of one of the following viruses as shown:

Virus	Subgroup of Virus	Number of hens	gs +albumens/ albumens tested
	Exogenous		
RAV-1	A	2	7/7
pRAV-1		1	4/4
tdPr-B	B	2	4/4
	Endogenous		
RAV-0	E	27	0/81
pRAV-0		2	0/15

pRAV-1 is virus recovered from RAV-1 proviral DNA cloned into the SacI site of pBr322. pRAV-0 is virus recovered from RAV-0 proviral DNA cloned into the SalI site of pBR322.

Albumens were collected from freshly layed eggs of the K28 hens which had been inoculated as above. Sera were collected from laying hens and virus was measured by assaying for the amount of particulate RNA-directed DNA polymerase. ALV group specific antigens (gs) were determined by assaying for complement fixation in reactions of egg albumen and

antiserum to gs antigens. The gs antigens in egg albumen had the electrophoretic mobilities that are characteristic of the viral proteins (p27, p19, p15, p12).

As indicated by the number of gs+ albumens found (gs+ albumens/albumens tested), exogenous avian leukosis viruses were congenitally transmitted, but endogenous were not.

Example 2: Congenital Transmission of Recombinants of Exogenous and Endogenous Avian Leukosis Viruses

K28 chickens were intravenously inoculated at 1 day of age with one of the following recombinants of exogenous and endogenous avian leukosis viruses:

Recombinant	Number of hens	gs + albumens/ albumens tested
NY201RAV-60	5	20/20
NY203RAV-60	1	4/4
NY202RAV-60	3	0/14
NY204RAV-60	1	4/4
NTRE-2	3	12/12
NTRE-7	5	0/15
recALVWF44, 45	3	0/11
recALVB22	4	10/10

Albumens were collected and assayed as in Example 1. Five of the eight recombinants underwent efficient egg transmission (NY201RAV-60; NY203RAV-60; NY204RAV-60; NTRE-2; recALVBr22) and three did not (NY202RAV-60; NTRE-7; recALVWF44,45). The recombinants were grouped according to whether they were shed into egg albumen or not.

Their genomes were then scored for markers for their endogenous and exogenous parents. Most markers were oligonucleotides that were diagnostic for the endogenous or the exogenous parents of the recombinants (i.e., markers for sequences in U5, p19, pol, gp37 and U_3). The electrophoretic mobility of the p27 protein was used as a marker for p27 and p27*. The subgroup of the recombinant was used as the marker for gp85. No markers were present for the p12 and p15 capsid proteins. (N, marker from endogenous parent; X, marker from exogenous parent).

Recombinant Virus	U5	p19	p27	pol	gp85	gp37	U3
			Transmitted				
NY201RAV-60	X	N+X	X	X	N	X	X
NY203RAV-60	X	X	X	X	N	X	X
NY204RAV-60	X	N+X	X	N	N	X	X
NTRE-2	N	N	X	X	N	N+X	X
recALVBr22	N	N	X	X	N	X	X
			Non-Transmitted				
NY202RAV-60	X	N+X	N	X	N	X	X
NTRE-7	N	N	N	N	N	N	X
recALVWF44,45	N	N	N	N	N	X	X

All of the recombinants had the subgroup E host range of their endogenous parent and U_3 sequence from their exogenous parent. Thus gp85 sequences that determine host range and U_3 sequences of ALVs do not determine whether ALVs undergo efficient congenital transmission. U5, p19 and gp37 sequences also do not appear to play a key role in congenital transmission.

There was, however, a direct correlation between the presence of the p27 marker of exogenous ALVs and the ability of a recombinant to be shed into egg albumen. The five recombinants with the exogenous p27 marker underwent efficient shedding into eggs and the three recombinants with the endogenous p27° marker did not.

Example 3 Construction, Testing and Suggested Field Protocol for Use of A Vaccine for Subgroup A Avian Leukosis Viruses

A. Vaccine Construction

The restriction endonuclease fragment bounded by the XhoI site at bp~630 and bp~5260 of RAV-0 proviral DNA was substituted for the comparable fragment in RAV-1 proviral DNA. Three such constructions were cloned in a derivative of the plasmid pBR322. Two of these constructions, designated Worcester Foundation 171 and 172 (WF171 and WF172), were transfected into turkey cells to produce a virus stock. The recovered virus has most of the gag and all of the pol sequences of the benign virus RAV-0 and the env and LTR sequences of the subgroup A avian leukosis virus RAV-1. The virus recovered from WF171 and WF172 grows well in cultured fibroblasts. WF82 includes a virus attenuated by the substitution of the 3' end and LTRs of RAV-0 for the comparable sequences of RAV-1. It is useful for testing the efficacy of viruses containing the U_3 sequences of an endogenous virus (RAV-0) as opposed to an exogenous virus (RAV-1) for vaccine production.

B. Testing of the Vaccine Virus - Production of Neutralizing Sera

Production of neutralizing sera in response to infection with RAV-1 (an exogenous ALV of subgroup A); virus recovered from WF171 and WF172; or virus recovered from WF82 was determined. As described above, virus recovered from WF171 and WF172 has most of the gag and all of the pol sequences of the benign virus RAV-0 and the env and LTR sequences of the exogenous subgroup A RAV-1. Virus recovered from WF82 has the 3' end and LTRs of RAV-0 substituted for the comparable sequences of RAV-1.

Approximately 1×10^6 infectious units (i.v.) of RAV-1 or of virus recovered from WF171 or WF172 were inoculated into the breast muscle of 4-6-week-old and 14-week-old K28 chickens. Production of neutralizing antibodies (antibodies that neutralize subgroup A ALV infection) was determined in sera from these chickens at 2 weeks and 4 weeks (and for RAV-1 inoculated chickens, also at 3 months) after inoculation. Results of these tests are shown in Table 1; results are based on data from 3-5 chickens per group. Titers of neutralizing activity are given as the logarithm of the dilution which inactivated at least 200 focus forming units (ffu) of RSV(RAV-1). NT in the Table represents not tested; NNA represents no neutralizing activity.

Approximately 1×10^3 or 1×10^6 i.u. of virus recovered from WF171, WF172 or WF82 were inoculated into the breast muscle of 10-week-old and 18-week-old K28 chickens. Production of neutralizing antibodies was determined in the sera from these chickens at 2 weeks and 4 weeks after inoculation. Results of these tests are shown in

Table 2; results are based on data from 1-4 chickens per group. Titers of neutralizing activity are expressed as described above.

The data from both these series of tests demonstrate that the viruses used (those recovered from WF171 and WF172) elicited a strong immune response in the inoculated chickens. The immune response was equivalent to that elicited by the exogenous RAV-1. In addition, these tests indicate that only small quantities of medium harvested from cultures infected with virus recovered from WF171 and WF172 are needed to inoculate chickens; that is, 1 milliliter of such medium (diluted 1:100) is sufficient to inoculate 1,000 chickens.

TABLE 1

Production of Neutralizing Sera in Response to Infection with Virus Recovered from WF171 and WF172

Virus	i.u.	Titer of Neutralizing Activity for RSV (RAV-1) at Different Times After Inoculation						
		4-6 weeks				14 weeks		
		2wk	4wk	3mo	8mo	2wk	4wk	3mo
RAV-1	10^6	2	3	3		NT	NT	NT
WF171	10^6	2	3-4			2-3	3-4	
WF172	10^6	2	3-4			2-3	3-4	
uninfected		NT	NNA			NT	NNA	

TABLE 2

Production of Neutralizing Sera in Response to Infection with Virus Recovered from WF171, WF172 and WF82

Virus	i.u.	Titer of Neutralizing Activity for RSV (RAV-1) at Different Times After Inoculation				
		10 weeks		18 weeks		
		2wk	4wk	3mo	2wk	4wk	3mo
WF171	10^6	2	3		2	3	
	10^3	2	3		NNA	2-3	
WF172	10^6	1-2	3		2	3	
	10^3	1-2	3		NNA	3	
WF82	10^4	NNA	NNA-2		NNA	NNA-1	
uninfected		NNA	NNA		NNA	NNA	

In addition, viruses were inoculated in the breast muscle of K28 chickens of the indicated ages. Between 2 and 4 chickens were in each test group. Presence of neutralizing antibody in yolks of eggs laid by the inoculated hens was determined. Titers of neutralizing sera were obtained in assays on one egg. In cases where assays were performed on more than one egg from a hen, the results were similar. Results are shown in Table 3. Titers of neutralizing activity are the logarythim of the dilution of sera which inactivated at least 200 ffu of RSV(RAV-1).

TABLE 3

Neutralizing Antibody in Yolks of Eggs Laid by Hens Inoculated with Virus Recovered from the WF170s

Virus	Age at Inoculation	Age at which neutralizing activity was determined: 5 months yolk	5 months albumen	8 months yolk	8 months albumen
Test 1					
RAV-1	4 wks	NT	NT	3	NNA
uninfected				NNA	NNA
Test 2					
WF171	14 wks	2-4	NNA		
WF172	14 wks	2-4	NNA		2132

C. Suggested Field Protocol for Use of Vaccine Viruses

The procedure for the use of virus recovered from WF171 and WF172 as a vaccine will be as follows:

1. Eggs to be hatched to establish a breeding population of hens will be screened to identify those which contain virus in their albumen. All eggs containing virus will be discarded.

2. Chicks hatched from virus-free eggs will be raised apart from other chickens.

3. At the time these chicks reach immune competence they will be vaccinated with virus recovered from WF171 or WF172.

After the initial year of vaccination, step 1 will no longer be necessary in the erradication program. Vaccination with virus recovered from WF171 or WF172 eliminates the Achilles heel of protocols now being tested by others - the re-establishment of congenital infections by the virus being used as a vaccine. See deBoer, G.F. et al., The control of lymphoid leukosis in a flock White Plymouth Rock chickens, The Veterinary Quarterly, 1:23-28 (1979); deBoer, G.F. et al., Horizontal transmission of lymphoid leukosis virus, influence of age, maternal antibodies and degree of contact exposure, Avian Pathology, 10:343-358 (1981).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. RNA comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus.

2. RNA of Claim 1 in which the endogenous retrovirus and the exogenous retrovirus are avian retroviruses.

3. RNA comprising gag sequences of an endogenous avian leukosis virus and sequences which encode antigens of at least one exogenous avian leukosis virus.

4. DNA comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus.

5. A vaccine including the DNA of Claim 4.

6. DNA of Claim 4 in which the endogenous retrovirus and the exogenous retrovirus are avian retroviruses.

7. A vaccine including the DNA of Claim 6.

8. DNA comprising gag sequences of an endogenous avian leukosis virus and sequences which encode antigens of at least one exogenous avian leukosis virus.

9. A vaccine including the DNA of Claim 8.

10. A constructed retrovirus having nucleic acid sequences comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus.

11. A vaccine including the retrovirus of Claim 10.

12. A retrovirus of Claim 10 in which the endogenous retrovirus and the exogenous retrovirus are avian retroviruses.

13. A vaccine including the retrovirus of Claim 12.

14. A constructed retrovirus having nucleic acid sequences comprising gag sequences of an endogenous avian leukosis virus and sequences which encode antigens of at least one exogenous avian leukosis virus.

15. A vaccine including the retrovirus of Claim 14.

16. A method of making DNA comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus, comprising the steps of:

a. producing DNA sequences comprising gag sequences of an endogenous retrovirus and DNA sequences which encode antigens of at least one exogenous retrovirus;

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle.

17. A method of Claim 16 in which the endogenous retrovirus and the exogenous retrovirus are avian retroviruses.

18. A method of making DNA comprising gag sequences of an endogenous avian leukosis virus and sequences which encode antigens of at least one exogenous avian leukosis virus, comprising the steps of:

a. producing DNA sequences comprising gag sequences of an endogenous avian leukosis virus and DNA sequences which encode antigens of at least one exogenous avian leukosis virus; and

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle.

19. A method of making a retrovirus having nucleic acid sequences comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus, comprising the steps of:

a. producing DNA sequences comprising gag sequences of an endogenous retrovirus and DNA sequences which encode antigens of at least one exogenous retrovirus;

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle;

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

20. A method of Claim 19 in which the endogenous retrovirus and the exogenous retrovirus are avian retroviruses.

21. A method of making a retrovirus having nucleic acid sequences comprising gag sequences of an endogenous avian leukosis virus and sequences which encode antigens of at least one exogenous avian leukosis virus, comprising the steps of:

a. producing DNA sequences comprising gag sequences of an endogenous avian leukosis virus and DNA sequences which encode antigens of at least one exogenous avian leukosis virus;

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle;

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

22. DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus.

23. DNA of Claim 22 in which the cis-acting signals are essential for an RNA transcript to undergo an avian retroviral life cycle.

24. DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus.

25. A vaccine including 1) DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus; and 2) DNA comprising gag sequences of an endogenous retrovirus and sequences which encode host range of an exogenous retrovirus.

26. A vaccine of claim 25 in which retroviral sequences are avian retroviral sequences.

27. A vaccine including 1) DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus; and 2) DNA comprising gag sequences of an endogenous avian leukosis virus and sequences which encode host range of an exogenous avian leukosis virus.

28. A constructed retrovirus having nucleic acid sequences which encode cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus.

29. A constructed retrovirus of Claim 28 in which the retroviral sequences are avian retroviral sequences.

30. A constructed retrovirus having nucleic acid sequences which encode cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus.

31. A vaccine including: 1) a constructed retrovirus having nucleic acid sequences which encode cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus and; 2) a constructed retrovirus having nucleic acid sequences comprising gag sequences of an endogenous retrovirus and sequences which encode host range of an exogenous retrovirus.

32. A vaccine of Claim 31 in which the retroviral sequences are avian retroviral sequences.

33. A vaccine including: 1) a constructed retrovirus having nucleic acid sequences which encode cis-acting sequences essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus and; 2) a constructed retrovirus having nucleic acid sequences

comprising gag sequences of an endogenous avian leukosis virus and sequences which encode host range of an exogenous avian leukosis virus.

34. A method of constructing DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for RNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus; and

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle.

35. A method of Claim 34 in which the retroviral sequences are avian retroviral sequences.

36. A method of constructing DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for RNA to undergo an leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus; and

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle.

37. A method of constructing a retrovirus having nucleic acid sequences which encode cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for RNA to undergo a retroviral life cycle and sequences which encode antigens of at least one virus other than a retrovirus;

b. linking the DNA sequences to encode an RNA that can undergo a retroviral life cycle.

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

38. A method of Claim 37 in which the retroviral sequences are avian retroviral sequences.

39. A method of constructing a retrovirus having nucleic acid sequences which encode cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for RNA to undergo an avian leukosis virus life cycle and sequences which encode antigens of at least one virus other than a retrovirus;

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle;

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

40. DNA comprising gag sequences of an exogenous avian leukosis virus; sequences which encode host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus.

41. A constructed retrovirus having gag sequences of an exogenous avian leukosis virus; sequences which encode the host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus.

42. DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein.

43. A constructed retrovirus having cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein.

44. A stock including: 1) a constructed retrovirus having gag sequences of an exogenous avian leukosis virus; sequences which encode host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus; and 2) a constructed retrovirus having cis-acting signals essential for an RNA transcript of DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein.

45. A method of making DNA comprising gag sequences of an exogenous avian leukosis virus; sequences which encode host range of an exogenous avian leukosis virus and pol sequences of an endogenous avian leukosis virus, comprising the steps of:

a. producing DNA sequences comprising gag sequences of an exogenous avian leukosis virus; sequences which encode host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus; and

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle.

46. A method of making an avian leukosis virus having gag sequences of an exogenous avian leukosis virus; sequences which encode host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus, comprising the steps of: a. producing DNA sequences comprising gag sequences of an exogenous avian leukosis virus; sequences which encode the host range of an exogenous avian leukosis virus; and pol sequences of an endogenous avian leukosis virus;

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle;

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

47. A method of making DNA comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian

leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein; and

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle.

48. A method of making an avian leukosis virus having cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein, comprising the steps of:

a. producing DNA sequences comprising cis-acting signals essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle; DNA sequences that control the production of an egg white protein; and DNA sequences that encode at least one nonviral protein; and

b. linking the DNA sequences to encode an RNA that can undergo an avian leukosis virus life cycle;

c. transfecting cells with the DNA; and

d. maintaining conditions suitable for the production of virus.

U3 R
R U5
LTR
U3 R U5
env
gp85
gp37
pol
gag
p15
p12
p27
p10
p9
KILOBASES
SEQUENCES FROM RAV-O
SEQUENCES FROM RAV-1
REGIONS IN WHICH VIRAL SEQUENCES ARE LINKED
Kb
pRAV-O
pRAV-1
pA
4889
1a
1b
2a
2b
2c
2147

FIG. 1

FIG. 2

SEQUENCES EXPRESSING AN ANTIGEN
SEQUENCES FROM RAV-O
SEQUENCES FROM RAV-I
REGIONS IN WHICH VIRAL SEQUENCES ARE LINKED
REGIONS IN WHICH VIRAL SEQUENCES AND X SEQUENCES ARE LINKED
SEQUENCES FROM THE TRANSCRIPTIONAL CONTROLS FOR EGG WHITE PROTEINS
SEQUENCES OF A PROTEIN TO BE EXPRESSED UNDER THE CONTROL OF OV
SEQUENCES FROM RAV-O
SEQUENCES FROM RAV-I
REGION IN WHICH VIRAL SEQUENCES ARE LINKED
REGIONS IN WHICH VIRAL, OV, AND Y SEQUENCES ARE LINKED
LTR
env
pol
gag
LTR
pX
pA
pAX
pOV
pY
pC
pCOVY
3a
3b
3c
4a
4b
4c
4d

FIG. 3

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/01352

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; A 61 K 39/21; C 12 N 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
IPC[4]	A 61 K C 12 N C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	Chemical Abstracts, volume 98, part 23, 6 June 1983, Columbus, Ohio, (US) H.L. Robinson et al.:"Cancer induction by insertional mutagenesis: the role of viral genes in avian leukosis virus induced cancers", see page 160, abstract nr. 192577m & Prog. Clin. Biol. Res. 1983, 119, 27-42	1-4,6,8,10, 12,14,16-21
X	Journal of Virology, volume 33, nr. 1, January 1980 P.N. Tsichlis et al.:"Recombinants between endogenous and exogenous avian tumor viruses: role of the C region and other portions of the genome in the control of replication and transformation", pages 238-249, see the whole document	1-4,6,8,10, 12,14,16-21
X,P	Science, volume 225, 27 July 1984, H.L. Robinson et al.:"New findings on the congenital transmission of avian leukosis	

* Special categories of cited documents: **

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th October 1985	08 JAN. 1986
International Searching Authority	**Signature of Authorized Officer**
4891 EUROPEAN PATENT OFFICE	G.L.M. Kruijzenberg

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	virus", pages 417-419, see the whole document	1-4,6,8,10, 12,14,16-21

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

The International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHED SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-21,25-27,31-33.

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/01352

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; A 61 K 39/21; C 12 N 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 61 K C 12 N C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 98, part 23, 6 June 1983, Columbus, Ohio, (US) H.L. Robinson et al.:"Cancer induction by insertional mutagenesis: the role of viral genes in avian leukosis virus induced cancers", see page 160, abstract nr. 192577m & Prog. Clin. Biol. Res. 1983, 119, 27-42	1-4,6,8,10, 12,14,16-21
X	Journal of Virology, volume 33, nr. 1, January 1980 P.N. Tsichlis et al.:"Recombinants between endogenous and exogenous avian tumor viruses: role of the C region and other portions of the genome in the control of replication and transformation", pages 238-249, see the whole document	1-4,6,8,10, 12,14,16-21
X,P	Science, volume 225, 27 July 1984, H.L. Robinson et al.:"New findings on the congenital transmission of avian leukosis	

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th October 1985	08 JAN. 1986
International Searching Authority	Signature of Authorized Officer
4893 EUROPEAN PATENT OFFICE	G.L.M. Nijsmeijer

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

virus", pages 417-419, see the whole document	1-4,6,8,10, 12,14,16-21

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE *

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim number ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING *

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHED SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-21, 24-27, 33-34

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM Form PCT/ISA/210 (suupplemental sheet(2))

1. Claims: 1-21,25-27,31-33 :

RNA, DNA and a constructed retrovirus comprising gag sequences of an endogenous retrovirus and sequences which encode antigens of at least one exogenous retrovirus, vaccines containing these and methods for their preparation.

2. Claims: 22-39 :

DNA, or a constructed retrovirus comprising cis-acting signals essential for an RNA transcript of the DNA to undergo a retroviral life cycle and sequences which encode antigens of at least one non-retrovirus and methods for their construction.

N.B. Claims 25-27 and 31-33 concern a vaccine comprising both subject 1 and 2

3. Claims: 40,41,44-46 :

DNA or a constructed retrovirus having gag sequences of an exogenous avian leukosis virus, sequences encoding host range of an exogenous avian leukosis virus and pol sequences of an endogenous avian leukosis virus and a method for their construction.

4. Claims: 42,43,44,47,48 :

DNA or a constructed retrovirus comprising cis-acting signal essential for an RNA transcript of the DNA to undergo an avian leukosis virus life cycle, DNA sequences that control the production of an egg white protein and DNA sequences that encode at least one non-viral protein and a method for making them.

N.B. Claim 44 concerns a stock containing subjects 3 and 4.